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Molecular phylogeny and status of *Diploicia* and *Diplotomma*, with observations on *Diploicia subcanescens* and *Diplotomma rivas-martinezii*

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Abstract: ITS rDNA sequence data shows that *Diploicia* and *Diplotomma* species form a monophyletic clade distinct from other *Buellia* species. This indicates that *Diplotomma* merits acceptance as a genus, and suggests that *Diploicia* should be treated as a synonym of *Diplotomma*, the earlier name. The data also shows *Diploicia subcanescens*, considered the fertile counterpart in a species pair with *D. canescens*, is nested within *D. canescens* and should be treated as a synonym despite reported chemical differences. In addition, the molecular data support the distinctness of *Diplotomma rivas-martinezii*, a species restricted to gypsum rocks in Spain, from the widespread *D. venustum*, which grows on calcareous rocks. Aposymbiotic cultures suggest that *D. rivas-martinezii* also differs from *D. venustum* in its germination and isolation success rates. One new combination is made: *Diplotomma pulverulenta* (Anzi) D. Hawksw. (syn. *Abrothallus pulverulentus* Anzi) for the lichenicolous species previously known as *Buellia pulverulenta*.
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Key words: *Buellia*, *Lecanorales*, lichens, *Physciaceae*, species pairs, Spain.

Introduction

Since the generic name *Diplotomma* Flotow (Lecanorales, *Physciaceae*) was resurrected as distinct from *Buellia* De Not. by Hawksworth *et al.* (1980), it has been adopted in numerous checklists and regional studies (e.g. Wei 1991; Purvis *et al.* 1992; Nimis 1993; Esslinger & Egan 1995; Filson 1996; Malcolm & Galloway 1997; Marbach, 2000; Eriksson *et al.* 2001; Llimona & Hladun 2001; Hafellner & Türk 2001; Coppins 2002). However, the name has not been universally accepted (e.g. Santesson 1993; Wirth 1995; Nordin 1996; Vitikainen *et al.* 1997; Diederich & Sérusiaux 2000; Scholz 2000) and the status of the genus has remained controversial.

However, in a study of the *Buellia* s. lat. species with pluriseptate ascospores, Nordin (2000) noted that *Diplotomma* 'might be used in a more narrow sense', as has been the case in the checklists accepting the genus referred to above, for species with calcium oxalate in the thallus, often pruinose apothecial discs, and a thick perispore; Purvis *et al.* (1992) also stressed the initially immersed ascomata, and the distinctive ascospores which they interpreted as distoseptate. However, the term 'distoseptate' has been used in different senses, as discussed by Nordin (1997), and is perhaps best avoided, but nevertheless the spores of *Diploicia* also have similarly thickened spore

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walls and septa developing secondarily recalling the polarilocular *Dirinaria*-type (Matzer *et al.* 1997). Nordin noted that the *Diplotomma* s. str. group was also distinct biologically, in that it included obligately or facultatively lichenicolous species. Interestingly, *Diploicia* Massal., which has a similar thallus structure and ascospores but a placodioid habit, has been unhestitatingly accepted by all authors in recent decades. However, the type species of the latter genus, *D. canescens* (Dickson) Massal., was included in *Diplotomma* when the generic name was first used (Flotow 1849, 1850). In order to check the affinities of *Diploicia* with respect to *Diplotomma* and also *Buellia* s. str., we examined several collections of the type species of these genera and further its supposed primary, generally fertile species *Dc. subcanescens* (Werner) Hafellner & Poelt. The latter has also been reported to differ chemically from the sorediate and generally infertile *Dc. canescens* (Elix *et al.* 1988). This topic is of particular interest since some presumed species pairs are proving to be single species when studied using molecular methods (Articus *et al.* 2002).

Buellia rivas-martinezii Barreno & A. Crespo (Follmann & Crespo 1975), a species name not mentioned by Nordin (2000), was transferred to *Diplotomma* by Molina & Crespo (2000). This species is a frequent and moderately polymorphic taxon that appears to be confined to crystalline gypsum rocks in dry conditions in the Meso-mediterranean bioclimatical level (Rivas-Martínez 1982). *Diplotomma rivas-martinezii* has a thin, pale grey or ochraceous, rimose or granular, K− thallus and lacks a dark prothallus. The apothecia are innate, abundant, rounded (0·8–1·2 mm diam.), and have an ephemeral thalline exciple which is normally K+ red; the apothecia later become slightly substipitate and somewhat convex (Barreno & Crespo 1975; sub *Buellia follmannii* Barreno & A. Crespo, non Dodge 1967). Probably due to the rareity of the biotopes where it grows, the species is not represented even in many large lichen herbaria; consequently it has not been included in revisionary studies of buellioid lichens (Scheidegger 1993; Nordin 1996, 2000). The species is related to *Dm. venustum* Körb. (Barreno & Crespo 1975; as *Buellia venusta*), a species discussed in detail by Nordin (2000), which has innate apothecia and usually a smooth or rimose, often subeffigurate thallus and frequently showing a peripheral black prothallus. Although in some collections the morphological and chemical differences between the two taxa are not unequivocal, the particular habitat of *Dm. rivas-martinezii* facilitates its identification. Based on chemical and morphological characters, Clauzade & Roux (1985), accepted *B. venusta* as an independent species with a K+ yellow thallus, but included *B. rivas-martinezii* as a synonym of *B. epipolia* (Ach.) Mong. with a K− thallus. Nordin (1996) noted that *B. epipolia* s. str. was a synonym of *B. alboatra* (Hoffm.) Th. Fr. and used the name *B. venusta* to include K+ and K− specimens.

The taxonomic level to be used for particular taxa is always open to different interpretations, but new and independent data sets, and especially molecular characters, may support one interpretation better than another. Additionally, molecular characters may give information about evolutionary pathways that can be used in hypotheses for the process of speciation. Features of the substratum have been considered as one important selective determinant of the presence of most lichen species. Lopez-Bilbao *et al.* (1996) demonstrated a differential gene expression depending on the substratum. However, there are no data relating this feature to the process of speciation. Although vegetative (production of soredia, isidia, or other dual propagules) *versus* sexual reproduction (production of basidiospores or ascospores) has been proposed as one evolutionary trend in lichens (Poelt 1970, 1972; Tehler 1982) no other hypothesis has been vindicated. Paraphyletic evolution is considered to be one important process by which the delimitation of new plant species may be explained (Rieseberg & Brouillet 1994; Vargas *et al.* 1998) although no model of paraphyletic speciation has been proposed amongst lichen-forming fungi.

TABLE 1. *Species included in the analysis, locations and reference collection codes for newly sequenced specimens, presence of intron products, and GenBank accession numbers*

Species	Locality (reference collection code†)	Intron present (+)	GenBank no.
1. *Diplotomma rivas-martinezii*	Morata-Titulcia, Madrid (MAF 7028)	—	AF352316
2. *Dm. rivas-martinezii**	Segarra, Cataluña (BCC 13365)	—	AF352317
3. *Dm. rivas-martinezii*			AF101286
4. *Dm. venustum*	Santos de la Humosa, Madrid (MAF 7029)	+	AF352319
5. *Dm. venustum*	Villalazán, Zamora (MAF 6227)	—	AF352318
6. *Dm. venustum*	Montblanc, Cataluña (BCC 13749)	—	AF352320
7. *Dm. alboatrum*			AF224351
8. *Diploicia canescens*	Cap de Creus, Cataluña (BCC 13752)	+	AF352321
9. *Dc. canescens*			AF250793
10. *Dc. canescens**	Castelló d'Empuries, Gerona (MAF 8657)		AF532168
11. *Dc. subcanescens*	Cap de Creus, Cataluña (BCC 13571)	+	AF352322
12. *Dc. subcanescens**	Port de Selva, Gerona (MAF 8656)	+	AF532167
13. *Buellia frigida*			AF276070
14. *B. disciformis*			AF250784
15. *B. elegans*			AJ421415
16. *B. zoharyi*			AJ421418
17. *B. georgei*			AF250787
18. *B. dijiana*			AF250788
19. *B. epigaea*			AF250785
20. *B. schaereri*			AF250871
21. *B capitis-regum*			AF250783
22. *B. insignis*‡			AF250790
23. *B. erubescens*			AF250786
24. *B. subdisciformis*	Cap de Creus, Cataluña (BCC 13750)	—	AF352323
25. *B. lindigeri*			AF250789
25. *Physconia grisea*	Casa de Campo, Madrid (MAF-Cub GPAR2)	—	AF542506
27. *Ph. grisea*			AF224368

*DNA extraction using DNeasy Plant Mini Kit (QIAGEN).

†BCC, Departament de Botánica, Facultat de Biologia, Universitat de Barcelona; MAF, Departamento de Botánica, Facultad de Farmacia, Universidad Complutense de Madrid.

‡Incorrectly identified as *Buellia papilla* in Grube & Arup (2001) and GenBank.

This study had three main objectives, to test: (1) the robustness of the generic status of *Diplotomma* s. str. in relation to *Buellia* and *Diploicia*; (2) whether *Diploicia canescens* and *D. subcanescens* are a species pair as has often been presumed; and (3) if *Diplotomma rivas-martinezii* is genetically distinct from *D. venustum*.

Material and Methods

Lichen material

Details of the material, area of collection, and location of reference specimens, are presented in Table 1. Specimens were air-dried and stored at room temperature. *Physconia grisea* (Lam.) Poelt was used as the outgroup.

DNA extraction

Total fungal DNA was extracted from the thalli according to the CTAB extraction method (Crespo *et al.* 1997; Cubero *et al.* 1999). Occasions when DNA extraction was carried out using the DNeasy Plant Mini Kit (QIAGEN) are indicated in Table 1.

PCR amplification

Amplification of the internally transcribed spacer region of the rDNA gene cluster was undertaken with the primer ITS_{1F}, as described by Gardes & Bruns (1993), or ITS_5 and ITS_4, as described by White *et al.* (1990). ITS_{1F} was designed to be specific for fungal sequences at the 3′ end of the small subunit gene of the

rDNA and overlaps with ITS_5, whereas ITS_4 has been described as a 'universal' primer and hybridizes at the 5' end of the large subunit gene (Gardes & Bruns 1993).

Amplification was carried out in 50 µl volumes consisting of 5 µl of 10 × DNA polymerase buffer (Biotools), 5 µl of 25 mM $MgCl_2$, 1 µl of a dinucleotide triphosphate (dNTP) containing 10 mM of each base, 2·5 µl of each primer 10 µM, 1·25 µl of a DNA polymerase (Biotools) and 8 µl of a 50-fold dilution of stock DNA. The PCR conditions were: denaturation 1 min at 94°C; annealing 1 min at 54°C; extension 2 min at 72°C (35 cycles); final extension 5 min at 72°C.

PCR products were purified with Biotools' PCR purification kit and sequenced, using the same primers, by the PRISM dRhodamine Terminator Candle sequencing Ready Reaction kit (Applied Biosystems) with detection on a 373A stretch automatic sequencing apparatus (Applied Biosystems). The total fragment was sequenced, comprising the 3' end of the small subunit gene, ITS 1, the 5·8S gene, ITS2 and the 5' terminus of the large subunit gene.

Sequence analysis

Complementary strands were compared with the assistance of Windows SeqMan (DNAStar) to check for reading errors and when possible, resolving ambiguities. Sequences were then aligned using Clustal X version 65b (Jeanmougin *et al.* 1998) and visually adjusted. Ambiguous aligned sites were removed, and the final alignment was used for the analysis. Maximum parsimony was applied to the data using the PAUP 4.0 program (Swofford 1999) with gaps being interpreted as missing data. The heuristic search option was used employing random addition of sequences and 100 replicates were generated with the tree bisection-reconnection (TBR) method of branch swapping, and the MulTrees options in effect. A neighbour-joining tree based on Kimura 2-parameter distances was also calculated using PAUP 4.0. Node reliability was assessed from 1000 bootstraps in both cases.

Isolation and culture of mycobionts

Isolated multispores were obtained from apothecia of *Diplotomma rivas-martinezii* (MAF 7028) and *D. venustum* (MAF 7029). The fungi were grown from discharged ascospores following Ahmadjian (1993). After germination, uncontaminated multispore isolates were maintained on BBM, pH 5·85 (Deason & Bold 1960) or transferred to 4% glucose LBM, pH 5·30 (Lilly & Barnett 1951) or malt yeast extract, pH 5·43 (1 l distilled water, containing malt extract 5·00 g, glucose 2·00 g, yeast extract 0·25 g). The medium contained 1·5% w/v agar. Simultaneously, enriched malt yeast extract was prepared by adding 40 ml of soil extract, at pH 5·80 (Esser 1976). Soil (500g) from the collection area were suspended in 1 l of bi-distilled water and boiled for 1 h. The supernatant was decanted, filtered, and made up to 1 l. The pH was checked and adjusted to pH 5–7 when necessary. As a stock solution, 100 ml of this extract were made up to 1 l with bi-distilled water and then sterilized. The inoculated plates were incubated at 18 ± 2°C, following Molina & Crespo (2000).

Results

DNA was extracted from all specimens and the rDNA target sequence was amplified. These PCR products were sequenced and their length differences (Table 1) were found to be due to a group I intron of approximately 200 bp inserted at the 3' end of the small subunit (1516). Previous reports of introns within lichen-forming fungi have identified a number of insertion sites within the ribosomal small subunit gene (DePriest 1994; Gargas *et al.* 1995).

The final data matrix contained 399 nucleotides after removing the intron, ambiguous sequences and alignments; 30·8% (123 nucleotides) were informative. Six trees which were equally parsimonious were obtained, each of 408 steps. The consensus parsimony tree obtained using this matrix (Fig. 1) had a consistency index (CI) of 0·822 and a retention index (RI) of 0·667 and reliably distinguished two monophyletic groups. One of them corresponds to *Buellia lindigeri* and the other one groups all remaining samples into two clades; one of these has two sister branches. The first (Fig. 1A) groups the *Diplotomma* s. str. species included (i.e. *Dm. rivas-martinezii*, *Dm. venustum*, and *Dm. alboatrum*) and also the species of *Diploicia* studied (i.e. *Dc. canescens*, and *Dc. subcanescens*). The other clade (Fig. 1B) groups all the remaining *Buellia* species, including the generally accepted type species *B. disciformis*. *Diplotomma* and *Diploicia* species are respectively monophyletic and sister groups with a reasonably robust bootstrap value (80%) in Fig. 1.

The distance tree (Fig. 2) shows a similar topology to the parsimony one. The *Diploicia canescens* and *Dc. subcanescens* collections were paraphyletic, and combined into a single clade with high bootstrap support (92%). In contrast, the specimens of *Diplotomma rivas-martinezii* formed a monophyletic clade with strong support

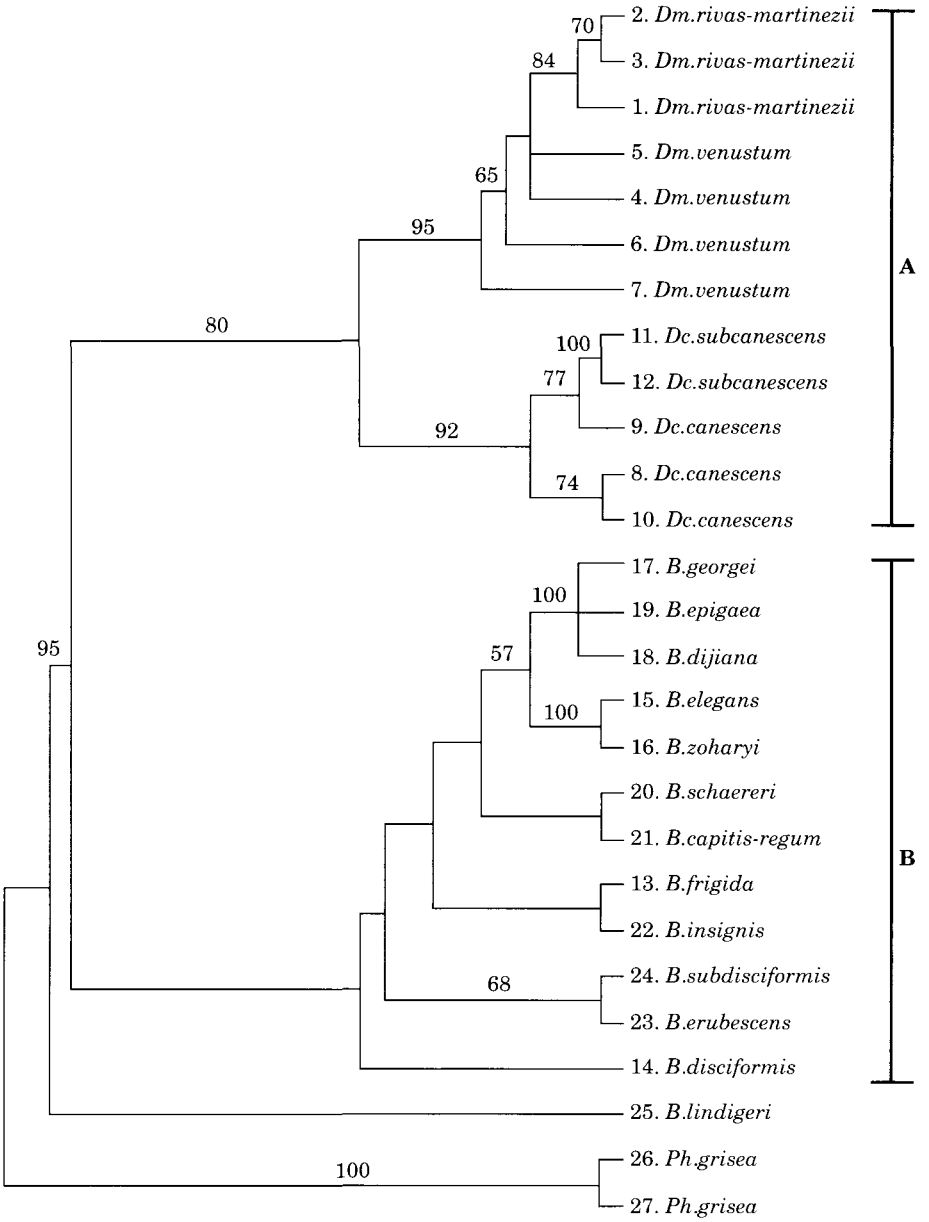

FIG. 1. Strict parsimony consensus tree. Branches are collapsed for bootstrap values of less than 50%. *Physconia distorta* was selected as the outgroup. *B.* = *Buellia*; *Dc.* = *Diploicia*; *Dm.* = *Diplotomma*.

(84%) and separate from collections of *Dm. venustum* which was not resolved as a monophyletic group itself.

When the cultured multisporic group of isolates from *Dm. venustum* was maintained on BBM for 4 months, a clear degeneration of the germinated hyphae was observed. These alterations, detected by optical microscopy, resulted in cell death on this medium—probably as a consequence of the

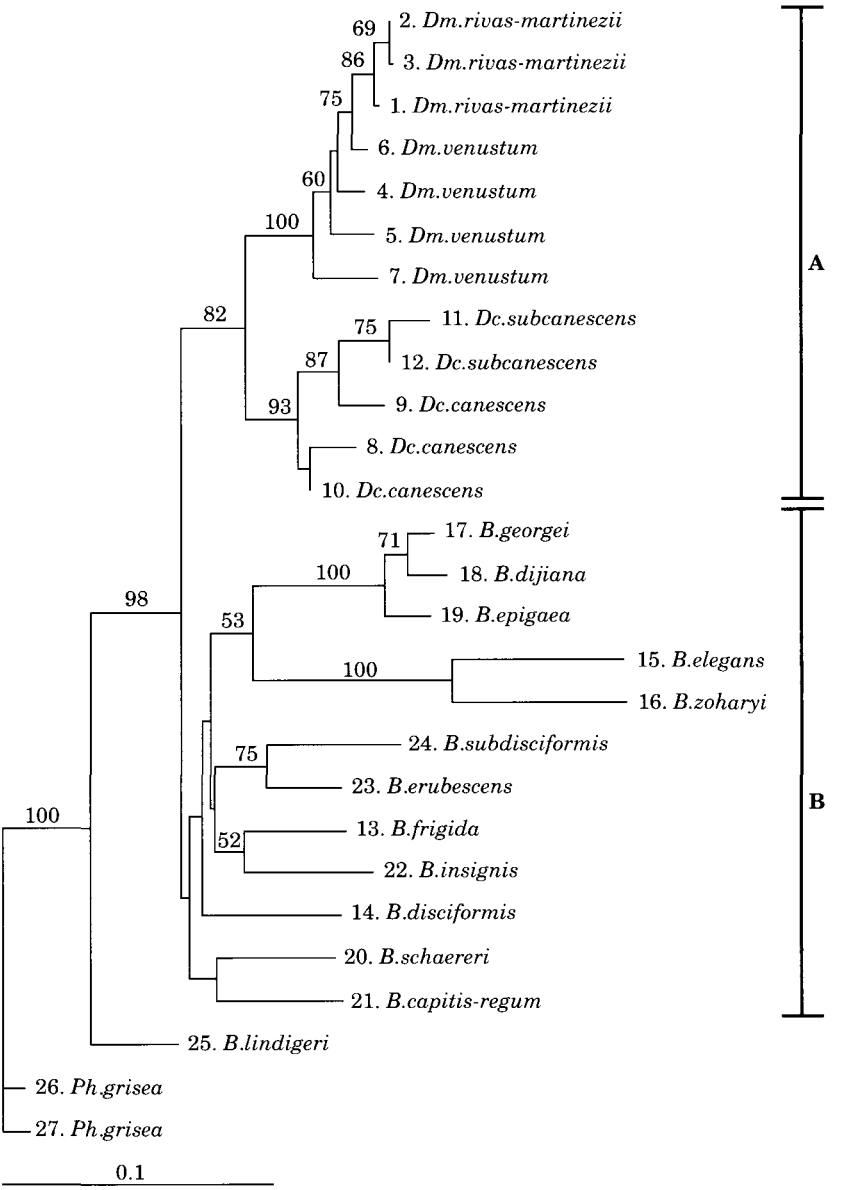

FIG. 2. Neighbour-joining distance tree. Bootstrap values over 50% are indicated. Scale bar indicates 0·1 Kimura-2 distance units. *B.* = *Buellia; Dc.* = *Diploicia; Dm.* = *Diplotomma.*

absence of a carbon source (Fig. 3A), since germinated isolated multispores transferred to 4% glucose LBM generated a thalline mycelium after 3 months of 2–3 mm diameter (Fig. 3B). *Diplotomma rivas-martinezii* developed through the first stages on BBM only. However, transfer of the multispores of the latter species to 4% glucose LBM resulted in collapse of the hyphae with structural alterations culminating in cell death (Fig. 3C). Surprisingly, a mycelium with a 95% success rate on inorganic medium was

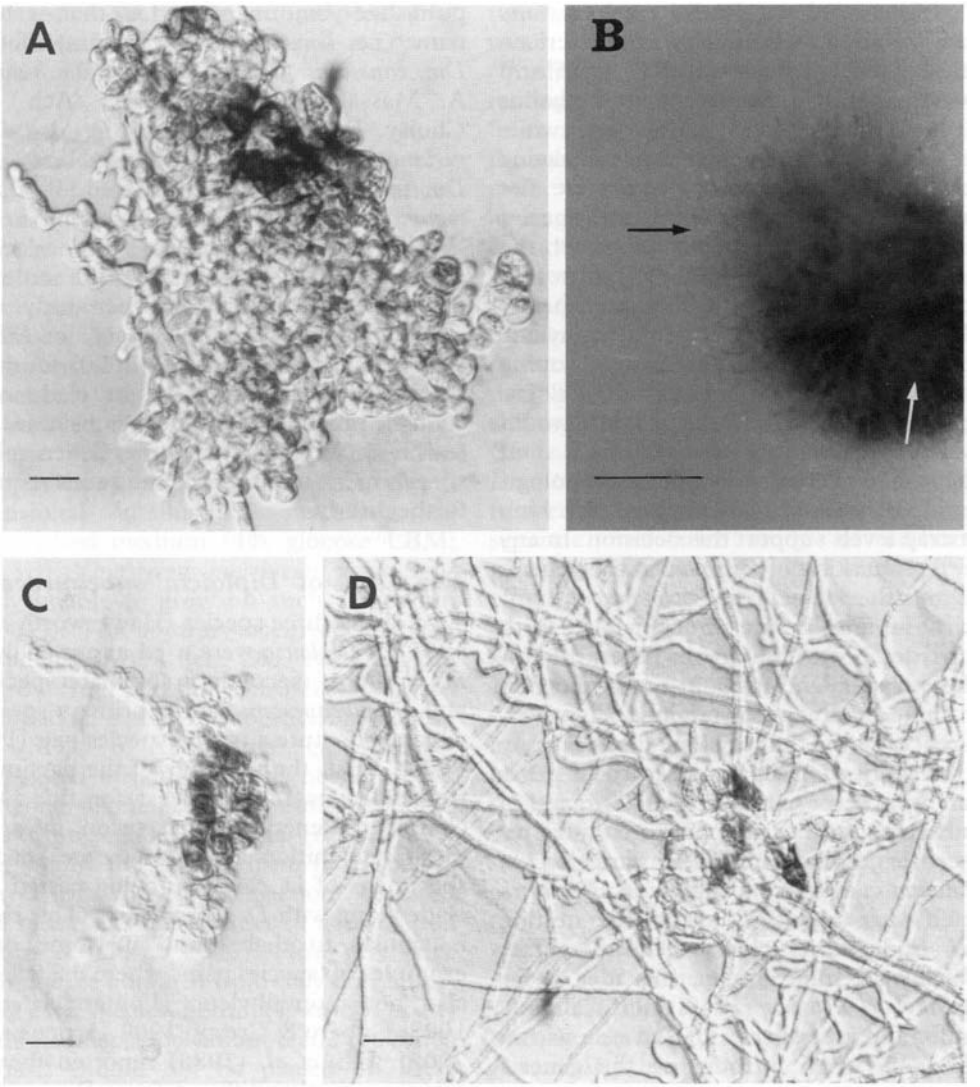

FIG. 3. Developmental stages of *Diplotomma* grown under axenic conditions. A, *Dm. epipolium* on BBM after 3 months; B, *Dm. epipolium* grown on 4% glucose LBM after 3 months (white arrow shows white superficial hyphae and black arrow indicates dark internal hyphae); C, *Dm. rivas-martinezii* on 4% glucose on BBM after 3 months; D, *Dm. rivas-martinezi* grown on BBM after 3 months. Scales: A, C & D=15 μm; B=0·2 mm.

observed 3 months after initial germination on that medium. These hyphae had a long interseptum distance and a low number of lipid drops, but structural alterations were not observed (Fig. 3D). Neither *Dm. rivas-martinezii* nor *Dm. venustum* grew on malt extract, with or without soil extract.

Discussion

Generic concepts

A phylogenetic hypothesis for evolution in the *Physciaceae* by Grube & Arup (2001), also based on ITS sequences, showed a clade where *Diploicia canescens* clustered

as the sister clade of *Buellia capitis-regnum* W. A. Weber, an endemic North American species with thick-walled muriform ascospores and a non-placodioid thallus (Nordin 2000: fig. 13). However, while Grube & Arup (2001) labelled this as having 'Diplotomma' ascospores, these were not defined and no *Diplotomma* s. str. species was included in their analysis. However, the results from our study (Figs 1 & 2) show that the *Diploicia* and *Diplotomma* s. str. species studied form a well-supported monophyletic group, a sister group of the clade grouping most of the other species of *Buellia* s. lat. (Fig. 1). Although a multigenic study would be ideal to confirm that *Diploicia* and *Diplotomma* are congeneric, the morphological and anatomical evidence and the high bootstrap levels support this decision. In any case, the retention of *Diploicia* at the generic level and the keeping of *Diplotomma* s. str. species in *Buellia* are indefensible. Both groups do, however, form separate monophyletic clades, and it could be argued that both should therefore be retained. However, differences in the thallus alone do not justify the recognition of separate genera in other lichen groups, for example *Caloplaca,* and in the absence of other supporting features we suggest extending the circumscription of *Diplotomma* s. str. to include *Diploicia* species with a placodioid thallus, uniting them under the oldest generic name, *Diplotomma.** Interestingly, this idea is far from new as Flotow (1849, 1850) already included the type species of *Diploicia* within *Diplotomma* when introducing the generic name.

Almost all species in this expanded concept of *Diplotomma* already have validly published binomials under that generic name (i.e. *Dm. alboatrum* (Hoffm.) Flotow, *Dm. canescens* (Dicks.) Flotow, *Dm. lutosum* A. Massal.†, *Dm. pharcidium* (Ach.) M. Choisy, *Dm. rivas-martinezii, Dm. scheidegeriana* (Bricaud & Cl. Roux) Nimis, and *Dm. venustum*). We do not include *B. capitis-regum* here in view of its grouping in the '*Buellia*' clade in our trees, and the lack of calcium oxalate in the thallus; the structure of the ascospores merits further study. One name change remains necessary, for *Buellia pulverulenta* which Nordin (2000) found to belong to the *Diplotomma* s. str. clade in his cladistic analysis based on non-molecular features‡. Whether some other lichenicolous species merit treatment in the genus requires further study.

The status of '*Diploicia*' *subcanescens*

Two of the three species (Hawksworth *et al.* 1995) of *Diploicia* were used in our analysis: *D. canescens* (secondary sorediate species), and *D. subcanescens* (fertile primary species) which constitute a typical species pair (Poelt 1970). First, the topology of the parsimony tree indicates that, at least for this DNA character, genetically the taxon does not merit recognition as two separate species, specimens of *D. canescens* being nested in a clade along with *D. subcanescens*. This result is similar to that found in some other examples of 'species pairs' where the taxa are also not monophyletic (Lohtander *et al.* 1998; Cubero & Crespo 1999; Articus *et al.* 2002). Elix *et al.* (1988) reported that *D. subcanescens* also differed from *D. canescens* in the presence of gyrophoric acid, but we do not consider that such a single unreplaced chemical warrants the recognition of such a chemotype as a species (Hawksworth 1976; Lumbsch 1998). Matzer *et al.* (1997) illustrated the ascospores and conidia of the two taxa which do not appear to differ

*The author citation given to the genus varies. It was first mentioned by Flotow (1849: 129) 'ad int.' But no description was provided. However, Flotow (1850) provided a detailed account with diagnostic features (: 380) and inclusion in a generic key (: 381–2) and the name was clearly validly published in that work. The generic name has sometimes been attributed to Massalongo (1852), as by Greuter *et al.* (1993), but Flotow has been cited as author by several recent workers (e.g. Nordin, 2000; Eriksson *et al.* 2001; Kirk *et al.* 2001) and we see no reason to dissent from that view.

†The correct name for *Buellia subdispersa* [syn. *Diplotomma subdispersum* (Krempelh.) Arnold] when placed in *Diplotomma* (Nordin 2000: 91).

‡**Diplotomma pulverulentum (Anzi) D. Hawksw., comb. nov.** (basionym: *Abrothallus pulverulentus* Anzi, *Cat. Lich. Sonder.*: 116, 1860; syn.: *Buellia pulverulenta* (Anzi) Jatta, *Syll. Lich. Ital.*: 400, 1900).

significantly. We therefore propose that *D. subcanescens* is treated as a synonym of *Diplotomma canescens.*

The status of *Diplotomma rivas-martinezii*

Some of the morphological differences reported between *Diplotomma rivas-martinezii* and *Dm. venustum*, for instance the colour or texture and habit of the thallus surface, may be due to different ecological conditions of the substrata or to local adaptations. However, the isolated fungal components of the two species differed and behaved oppositely when cultured on inorganic and organic media. While *Dm. venustum* grew and developed a three-dimensional 'thallus-like' mycelium on the enriched medium (4% glucose LBM; Fig. 3B), *Dm. rivas-martinezii* was completely unable to grow on such a medium (Fig. 3C). The contrary occurred on basal medium (BBM), where *Dm. venustum* did not grow (Fig. 3A) but *Dm. rivas-martinezii* developed an extensive superficial mycelium (Fig. 3D). In the latter case, three-dimensional structures were not observed and hyphal diameter and the number of lipid drops were smaller (Molina *et al.* 1997). Additionally, the isolates from both species differed with respect to ejection, development rates, and the manner of germination (Molina & Crespo 2000). However, since cultures were obtained from only one collection of each species, nothing is known of any possible infraspecific variation and too much emphasis should not be placed on these observations. Nevertheless, such distinct behaviours suggest that different genetically determined biological or physiological patterns occur at least between the two samples cultured.

The molecular analysis of ITS sequences (Figs 1 & 2) showed that the three samples of *Dm. rivas-martinezii* from gypsaceous substrata in different geographical regions were monophyletic (Fig. 1B). Samples of *Dm. venustum* collected near *Dm. rivas-martinezii* localities were placed further apart in the phylogenetic tree (for example, the two sites of *Dm. rivas-martinezii* and *Dm. venustum* from Madrid are separated by no more than 30 km). In evolutionary terms (Figs 1 & 2) *Dm. rivas-martinezii* could be a monophyletic species more recently derived from a population of *Dm. venustum*. Although the bootstrap values of 65% (maximum parsimony) and 60% (neighbour joining) are too low to have the highest confidence in this result, a paraphyletic relationship is suggested in relationship to *Dm. alboatrum*. However, the three samples of *Dm. venustum* studied were not monophyletic. A number of taxa have been named morphologically as close to *Dm. venustum* and/or *Dm. alboatrum* (cf. *B. lainea* in Clauzade & Roux 1985; synonymized with *Dm. alboatrum* by Nordin 2000) and any further taxonomic conclusions require a separate molecular study of additional populations. Genetic isolation leading to apparently cryptic species has been reported in an increasing number of cases in free-living and lichen-forming fungi (Crespo *et al.* 1999; Hawksworth 2001). Even in the event of *Dm. venustum* being considered an heterogeneous taxon, the topology of the phylogenetic tree nevertheless supports the retention of *Dm. rivas-martinezii* as an independent taxon. The results from the preliminary culture experiments suggest that physiological characteristics may reinforce this interpretation. Moreover, the ITS region has proved to be a good species-level marker for lichens (Groner & LaGreca 1997; Crespo & Cubero 1998). Consequently these results justify the retention of *Dm. rivas-martinezii* at the level of species.

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